Variable Oxygen Exposure Causes Preretinal Neovascularization in the Newborn Rat

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Purpose. To test the hypothesis that variable hyperoxia potentiates preretinal neovascularization in newborn rats, and to establish a more reliable animal model of ROP in which therapies designed to inhibit abnormal angiogenesis can be tested.

Methods. Immediately after birth, litters of Sprague Dawley albino rats and mothers were placed in an incubator containing 40% oxygen. After 12 hours, the oxygen was increased to 80% with a transition time of less than 1 min. For the ensuing 7, 10, or 14 days, the oxygen was alternated between 40% and 80% every 12 hr in a stepwise fashion. Other litters were kept in constant 80% oxygen or in room air for the same three time periods. After exposure, rats were either killed or placed in room air for an additional 2, 4, or 7 days before being killed.

Results. When rats were killed immediately after oxygen exposure, the resulting vessel loss in rats exposed to 40%/80% oxygen was identical to that of animals exposed to 80% (vessels constituted 12.2 ± 2.2% of total retinal area in cyclic oxygen vs 12.0 ± 1.2% in constant oxygen). However, preretinal neovascularization subsequently occurred in 66% (63/96) of all rats exposed to cyclic oxygen followed by a room air period but in no rats (0/50) exposed to constant oxygen followed by room air. Preretinal vascular proliferation consisted of glomerular tufts of endothelial cells, or mature, lumenized vessels containing red blood cells.

Conclusions. Consistency of oxygen therapy is more important than overall oxygen level in inducing retinopathy. Consideration should be given to tighter control of intended oxygen therapy in premature infants, regardless of the target saturation level. Invest Ophthalmol Vis Sci. 1993;34:576-585.

Advances in neonatal intensive care have caused a second "epidemic" of retinopathy of prematurity (ROP) that has paralleled the steadily increasing survival rate of premature infants. It is estimated that 3400 infants will suffer visual impairment from this condition each year, while 650 will be blinded. One corollary to the increased survival of preterm babies is a relative scarcity of human retinal tissue available for research. To examine the pathogenesis of ROP in a well-controlled fashion, it is necessary to return to the same strategy as that used during the 1950s—scrutiny of the effects of elevated oxygen on the retinas of newborn animals. The rationale for this strategy was articulated by Gole and it remains unchanged: "The [retinal] cells of a sick [premature] infant suffering from altered perfusion, metabolic acidosis and respiratory failure may . . . be unable to cope with a level of arterial oxygen which would be otherwise inoffensive. Because the experimental animal is essentially healthy and without intrinsic respiratory or metabolic disturbance, there are relatively few . . . factors to confuse the picture in the laboratory, where only one experi-
mental variable is altered—the inspired oxygen concentration.\textsuperscript{11} The use of animal models remains valuable, in part, because of a variety of powerful techniques developed since the early experiments with hyperoxic animals in the 1950s.

ROP is a disease that manifests itself in two characteristic stages: an initial period of retinal vascular attenuation that occurs during an infant's oxygen therapy, and a subsequent stage of vasoproliferation that is promoted by the infant's removal to room air.\textsuperscript{4-7} The initial attenuation appears to be caused by at least two, and perhaps three, factors: vasoconstriction, developmental retardation of new vessels, and, possibly, destruction of some existing vessels. The second (proliferation) stage is thought to be stimulated by retinal ischemia.\textsuperscript{8} This is the expected result of previous oxygen-induced vessel attenuation in combination with a lower level of inspired oxygen after the infant's transition to room air.

We have chosen the rat model of ROP to test this theory of pathogenesis. Historically, this model has been criticized because of its relative inability to demonstrate preretinal neovascularization after hyperoxic exposure.\textsuperscript{3} However, past experiments with rats have always employed constant oxygen exposures. Under these conditions, the initial stage of retinal vascular attenuation may be observed in the absence of the later proliferative stage.\textsuperscript{9} This report describes a successful attempt to consistently induce preretinal neovascularization in oxygen-exposed newborn rats. We are convinced that our success stems directly from a unique protocol of systematic variation of oxygen level during the exposure.

Preretinal neovascularization is the critical initial step in the chain of events that results in retinal detachment and blindness in infants. The ability to cause it consistently in animals is a significant step in studying the pathogenesis of ROP and, furthermore, it is the first step necessary to the design of novel therapies for infants. The significance of an experimental design that uses variable oxygen exposures, and the implications of this design for neonatal care, will be discussed.

**MATERIALS AND METHODS**

**Exposure Protocol**

Immediately after birth, litters of Sprague Dawley albino rats were placed with their mothers in an oxygen incubator (modified Isolette). After an initial 12-hr exposure to 40% oxygen, the oxygen level supplied to the animals was rapidly increased to 80%. For the next 7, 10, or 14 days, oxygen levels were altered between 40 and 80% every 12 hr in a stepwise fashion (Fig. 1). Other groups were maintained for the same lengths of time, in either constant 80% oxygen ($n = 63$) or room air ($n = 53$). At least four measurements of oxygen level were taken at random times on each day of the exposures; measured oxygen levels were not observed to vary by more than \( \pm 2\% \) during any experimental period. After the exposure, some rats were killed, while the remaining animals were moved to room air for an additional 2, 4, or 7 days before being killed. Mothers were fed rat chow ad libitum. The experimental animals were maintained under 12L:12D lighting of 200 lux, with the light cycle offset by 6 hr from the oxygen cycle.

After being killed, rats were enucleated; one retina was dissected and flat mounted for ADPase staining and the fellow eye was processed for cross-sectional histologic examination. A few rats were randomly chosen for ink-perfusion to assess overall vessel

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{This graph represents the oxygen level over the course of a typical experiment. At no time was the oxygen observed to deviate by more than 2\% from the intended level throughout the length of the exposure. The postexposure room air environment (20.9\% oxygen) is illustrated by a dashed line. Vertical arrows designate the three times at which animals were removed from the incubator to room air. Length of stay in room air before being killed is shown in Table 1.}
\end{figure}
density. In all, 239 rats were employed in these experiments (186 oxygen-exposed and 53 room-air controls). All investigations conformed to the ARVO Resolution on the Use of Animals in Research.

Ink Perfusion
Deeply anesthetized rats were perfused through the left ventricle with 5–7 ml. of India ink. Eyecups were removed and retinas were dissected, marked for orientation, and dried on microscope slides. The resulting flat mounts were covered with glycerin:PBS (1:1) and glass coverslips, and then photographed. Analysis of the integrity and extent of retinal vasculature in the flat-mounted retinas was accomplished by an image analysis system consisting of an Olympus SZ40 dissection stereomicroscope with advanced transmitted light base (Delta Optical Instruments Co., Little Rock, AR), a DAGE-MTI CCD-72 series digital camera (DAGE-MTI, Inc., Michigan City, IN), Neotech Image Grabber frame capture hardware (Neotech Ltd., Hampshire, UK), and Enhance image enhancement and analysis software (MicroFrontier, Des Moines, IA). The density of retinal vessels was calculated from a computer-derived binary image by dividing the number of pixels that represented vessels within an area of the retina by the total number of pixels within the retinal area. Measurements were expressed as percent of retinal area constituting vessels. Examples of the binary images are illustrated in Figure 2.

ADPase Staining
Retinal vasculature was examined by a modification of the lead sulfide technique of Flower et al. 

Eyes were enucleated, corneas were removed, and the remaining eyecups were placed in 10% neutral-buffered formalin (NBF, pH 7.4) on ice for 7–10 min. Retinas were excised in cold phosphate-buffered saline (PBS, pH 7.4), flattened, and stored overnight at 4°C in NBF. They were then washed (5 × 15 min) with 50 mM tris maleate buffer (pH 7.2) at 4°C before incubation. ADPase incubation medium contained 3.0 mM lead nitrate and 6.0 mM magnesium chloride (Sigma Chemical Co., St. Louis, MO) in 0.2 M tris maleate buffer (pH 7.2). The solution was made fresh, filtered, and warmed to 37°C. Just prior to placing a retina into the incubation solution, 10 mg of ADP (adenosine 5'-diphosphate, grade VI crystalline di[monocy-clo-hexylammonium] salt; Sigma Chemical Co., St. Louis, MO) was added to 10 mL of solution. The retina was incubated for 15 min with gentle agitation. It was then washed thoroughly in tris maleate buffer (five changes at room temperature), after which it was placed in a 1:10 dilution of ammonium sulfide (light solution; Fisher Scientific, Silver Spring, MO) for 1 min. The retina was then washed three times in tris maleate buffer. Retinas were mounted on a microscope slide with 1:1 PBS and glycerin for viewing as flat mounts. After locating areas of interest in ADPase-stained flat-mounted retinas, some were removed from slides and placed in 2% glutaraldehyde to begin processing for cross-sectional histology.

Light Microscopy
Whole eye cups (or previously flat-mounted retinas processed as described) were submerged in 2% glutaraldehyde in 0.1 M PIPES (pH 7.4) and stored over-
night at 4°C. Tissue was post-fixed in 1% OsO₄, dehydrated in ethanol, and embedded in epon. Thick sections (0.5 μm) were taken at 50-μm intervals from the retinal periphery to the optic nerve and stained with 2% Toluidine Blue; one-half of each retina, from the ora serrata to the optic nerve head, was sectioned. Observation of ADPase-stained retinas had confirmed that the sites of oxygen-induced pathology were randomly disbursted throughout the mid-periphery to the far periphery of all four retinal quadrants. For this reason, the direction of sectioning was not considered critical; therefore, the sectioned retinas were randomly oriented with respect to their radial symmetry. Because of rapid growth of the eye during this stage of development, the number of retinal cross-sections obtained from a given retinal hemisphere varied somewhat with age. However, the same number of sections from both oxygen-exposed and control rats was assessed for each timepoint.

In the context of this study, neovascularization is defined as the presence of preretinal cellular proliferations whose origin from existing retinal vessels could be demonstrated. Neovascularization was reported only in those cases in which cell or tissue outgrowth penetrated the inner limiting membrane in retinal cross-sections. Generally, affected rats displayed bilateral pathology; however, unilateral preretinal growth was considered to be sufficient reason to warrant a positive notation for a given animal. Evidence obtained from flat-mounted retinas was not considered conclusive.

**RESULTS**

In retinas examined immediately after removing rats from the constant or variable oxygen environments, ink perfusion demonstrated attenuation of the retinal vasculature compared to that of the retinas of age-matched, room air-raised rats. For each of the three variable oxygen exposure periods (7, 10, or 14 days), the loss of vessels was no different than that caused by exposure to constant oxygen for the same length of time. In other words, ink-perfusion indicated that both the variable and the constant oxygen exposures affected the retina identically with respect to vessel loss during hyperoxia. A comparison of digitized images of ink-perfused retinas from constant and variable oxygen for the 14-day exposure is illustrated in Figure 2. Vessels composed 12.0 ± 1.2% of the retinal area in rats exposed for 14 days to constant 80% oxygen and 12.2 ± 2.3% of the retinal area of rats exposed to variable 40/80% oxygen. Smaller values were calculated for 7- and 10-day exposures. At no exposure duration was there a statistically significant difference (P < 0.05) between constant and variable oxygen treatments.

No neovascularization was observed in any retina of rats killed immediately after removal from constant or variable oxygen (n = 13 and n = 27, respectively). Constant oxygen treatment did not induce abnormal neovascularization (preretinal or otherwise), regardless of the length of the postexposure period (n = 63). Variable oxygen exposure, on the other hand, induced preretinal neovascularization in 66% of all rats exposed for 7, 10, or 14 days followed by 2, 4, or 7 days in room air (n = 96). The incidence of preretinal neovascularization induced in each variable oxygen treatment group is presented in Table 1.

TABLE 1. Incidence (Percent) of Preretinal Neovascularization in Rats Exposed to Variable Oxygen

<table>
<thead>
<tr>
<th>Oxygen Exposure* (days)</th>
<th>Room Air Postexposure (days)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>7</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>0 (0 of 10)</td>
<td>50 (5 of 10)</td>
<td>67 (6 of 9)</td>
<td>50 (5 of 10)</td>
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</tr>
<tr>
<td>10</td>
<td>0 (0 of 10)</td>
<td>67 (6 of 9)</td>
<td>71 (5 of 7)</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>0 (0 of 7)</td>
<td>71 (10 of 14)</td>
<td>81 (17 of 21)</td>
<td>56 (9 of 16)</td>
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</tr>
</tbody>
</table>

* Variable exposure to the 40%/80% oxygen regime, with changes every 12 hr. No rats exposed to constant 80% oxygen developed preretinal neovascularization.

On removal of 40/80% oxygen-exposed rats to room air, features of retinal vasoproliferation included both normal and abnormal vessel develop-
An acceleration of superficial and deep capillary bed development began immediately on exposure to room air. New capillary growth was relatively normal, except at the interface with the peripheral avascular retina, where a narrow band of vessels formed a thin veil of fibrous extensions (Fig. 3). By day 2 in room air, additional outgrowth of vascular material into the vitreous occurred in the form of tufts or sheets of endothelial cells (Fig. 3). The tufts appeared to be hyperplastic and contained an abundance of ADPase-positive cells (Fig. 4A). In cross-section, their cellular composition consisted primarily of typical vascular endothelial cells, but few, if any, patent vessels (Fig. 4B). The sheets of vascular material (Fig. 5A) were often attached to the retina in more than one location (Fig. 5B).

By day 4 in room air, preretinal growth frequently consisted of masses of endothelial cells that extended from the plane of the superficial surface of the retina. In contrast to day 2, areas of canalization with occasional mature, luminized vessels that often contained red blood cells had developed (Fig. 6). The sequence

FIGURE 4. The ADPase-stained abnormal vascular growths often resemble glomeruli or round balls of endothelial cells (A). Original magnification is ×560. In cross section (B), these endothelial tufts can be distinguished as hyperplastic growths that reside in a more superficial plane than do the vessels from which they extend. (Original magnification ×1550.)
of their appearance indicated that tufts or sheets of uncanalized endothelial cells were produced first at the retinal surface, presumably as extensions of pre-existing superficial vessels. These proliferative elements then formed disorganized cords, canalized, and differentiated into mature vessel loops or bundles. In cases where the plane of section passed through the location of retinal attachment, the origin of the preretinal growth was confirmed to be the existing retinal blood vessels (Fig. 7).

During the 4-day postexposure period, degeneration of retinal neurons was evidenced by sporadic pyknosis in the inner nuclear layer in all three variable exposure groups (Fig. 8). The superficial vessels had continued their normal advance and extended approximately 80–90% of the distance to the ora serrata in the 14-day exposure group, while the deep net lagged slightly behind. Although the rat retina contains a dense complement of spindle-shaped vascular precursor cells at this stage of development, no alteration of their number, morphology, or role in the vasogenesis process was evident. The growth of neovascular tufts did not appear to involve these precursors; instead, abnormal vessels appeared to grow by budding of existing vessels of the superficial vascular net.

The most severe cases are exemplified by Fig. 9. This low-magnification photograph of a retina from a rat exposed to 14 days of variable oxygen followed by 4 days in room air shows a retina that contained a ridge of neovascular growth extending over nearly one-half
of the retinal circumference in the mid-periphery. Beneath this preretinal growth, the retina thickened significantly, but without obvious clumping of vasoformative cells. This neovascular growth typically arose just posterior to the peripheral-most extension of retinal vessels. The vessels anterior to this ridge of neovascular growth took the form of a veil-like border along the peripheral avascular retina.

No evidence of tractional force exerted by the preretinal vessels on the retina was observed, nor was there any evidence of retinal detachment in any treatment group. However, retinal folds were observed in the majority of rats exposed to constant or variable oxygen followed by room air (illustrations of such folds, and a discussion of their significance, can be found elsewhere). The fact that retinal folds without abnormal neovascularization occurred in rats exposed to constant oxygen, in the absence of abnormal neovascularization, indicates that the folds probably result from dysplastic retinal development. In several cases, the dysplasias were quite elaborate and consisted of rosettes of the outer retinal layers. None of the control rats demonstrated retinal folds or rosettes. Little difference in the morphology or severity of retinal pathology was seen between the 4-day and 7-day postexposure periods. However, there was a reduction in the overall incidence of neovascularization at the 7-day postexposure timepoint that may have resulted from spontaneous resolution of the pathology. The degree of pyknosis in the inner nuclear layer was unchanged after 3 additional days in room air.

**DISCUSSION**

**Experimental History**

Isolated reports of preretinal neovascularization in oxygen-reared rats appeared as early as Patz's 1954 study. It should be noted that he was unable to reproduce this finding later. Abnormal vasoproliferation in newborn rats maintained in room air after a...
Preretinal Vessels in the Hyperoxic Rat

FIGURE 8. A cross-sectioned retina from an animal raised in variable oxygen for 14 days exhibits marked pyknosis of the inner nuclear layer. This example represents the worst case of degeneration of the inner retina observed. (Original magnification X320.)

period of oxygen exposure was also described by Brands et al. Ashton and Blach questioned the claims of these two investigative teams, describing them as “inadequately substantiated on the evidence provided.” As a result, the rat lost favor as an animal model for retinopathy of prematurity; its usefulness has only recently been reexamined.

Since 1988, preretinal neovascularization in rat models of ROP has been published in three reports. Ricci and Calogero described “marked peripheral retinal neovascularization” in rats raised in 80% oxygen for the first 5 days of life followed by 5 additional days in room air. However, the photographic evidence supporting their observations of retinal vasoproliferation was based on ink-perfused, flat-mounted retinas. In our opinion, this technique is not reliable as the sole determinant of vascular integrity. Cross-sectional retinal histology was used to identify extraretinal neovascularization by Ventresca et al. Their experiments involved 40 newborn rats, half of which were exposed to 80% oxygen for 10 days, followed by room air for 15 days. Preretinal neovascularization was found in 16 of the 20 rat pups. Reynaud et al. presented evidence of preretinal neovascularization in rats raised in 80% oxygen for 6 days followed by room air for 11 days using the ADPase staining approach developed by Flower et al., but no mention was made of the incidence of neovascularization in their study population.

Constant Versus Variable Oxygen

We were unsuccessful in reproducing these findings until we instituted a regimen of systematically varied oxygen. This may, in fact, explain the inconsistencies in past studies; perhaps investigators who meticulously maintained constant ambient oxygen levels throughout the exposure saw no neovascularization, while those who were less concerned with oxygen level consistency were able to produce new vessels.

Constant oxygen levels do not necessarily reflect
the situation in the neonatal ICU. Indeed, in this setting, sick infants who suffer from acute pulmonary distress and associated anoxia actually receive a highly variable course of oxygen therapy, in spite of carefully orchestrated alterations in the prescribed oxygen administration that are designed to maintain a constant saturation level. Unfortunately, there is a time lag between the point at which an infant’s oxygen saturation falls below the predetermined critical level and the time at which increased oxygen delivery results in an equilibrated return to desired therapeutic levels. Our theory that variable oxygen may promote preretinal neovascularization is supported by the fact that these sick infants are the most likely to develop severe retinopathy.19,20

We propose that the consistency of oxygen delivery is more important than the overall oxygen level in inducing retinopathy. In fact, we have demonstrated that the retinas of rats maintained on a 40/80% cycle of oxygen were more severely affected than those of rats maintained at 80% alone, although the latter received considerably more inspired oxygen over the exposure period. Furthermore, ink perfusion evaluations showed no difference in the degree of vasoattenuation caused by the two exposures. This puzzling observation appears to contradict the theory that overall retinal ischemia upon removal to normoxia is the single cause of abnormal vessel growth. What is the means by which variable oxygen might preferentially induce abnormal neovascularization? Perhaps a variation of the ischemia/reperfusion theme in this role is worth considering.

Ischemia/Reperfusion

Ischemia/reperfusion carries an inherent component of episodal hypoxia. In our experiments, episodes of relative hypoxia are the expected result of changes in inspired oxygen, rather than reduced blood flow. Reduction of ambient oxygen from 80% to 40% would signal the onset of retinal ischemia in this scenario, while the step up from 40% to 80% would correspond to reperfusion. Ischemia alone can cause retinal tissue damage, but ischemic retinal tissue suffers additional damage when reperfused. Reactive oxygen species are often implicated in the injury induced by reperfusion of ischemic tissue.21 The retina is highly susceptible to attack by these molecules, especially during development, when it has not formed its normal adult complement of defense mechanisms.22 Furthermore, the probable presence of oxygen-derived free radicals in the retina is enhanced by elevated inspired oxygen. In addition to the possible damage mediated by these molecules, it has been indicated that other insults play a causal role in reperfusion injury, including stimulation of platelet-activating factor, release of lysophosphatides, and disturbances of membrane ion conductances.23 If any of these insults was present during these experiments, an effect on retinal neurons would be expected. Nuclear pyknosis, particularly of inner retinal neurons, is recognized to be the result of ischemia/reperfusion injury.21 Pyknosis of the inner nuclear layer was observed in a substantial number of retinal sections from rats raised in variable oxygen. Although some pyknosis of the inner retina occurred in rats raised in constant oxygen, these pyknotic nuclei were limited to the regions immediately surrounding dysplastic folds and rosettes.

A recent report of a direct link between retinal ischemia and cell proliferation, including induced mitosis of vascular cells, is pertinent to this discussion.24 This paper indicated that “ischemia itself, or one of its components such as hypoxia, stimulates the cell proliferation either directly or . . . through a chemical intermediary.” The authors point out that some proliferative retinopathies, such as diabetic retinopathy and branch vein occlusion retinopathy, carry a component of ischemia, as evidenced by fluorescein angiography. Although we did not conduct angiograms on rats in this study, we have previously demonstrated retinal ischemia in angiograms from newborn rats raised in constant oxygen.25 A comparison of angiograms with postmortem ink-perfusion of the same eye revealed crucial differences in the two methods, particularly in their abilities to illustrate the extent of vessel patency under physiologic conditions. The ink-perfused retinas in Figure 2 may not adequately measure retinal ischemia. Angiographic assessment is necessary for a valid comparison of vessel patency and ischemia in variable and constant oxygen. For this reason, we are not prepared to abandon the theory that retinal ischemia is the primary force behind vasoproliferation.

After decades of research into the cause of ROP, animal models remain important. The common ground linking the models to the human infant is the early manifestations of the disorder—vaso-obliterration and neovascularization. One important aspect of this model is that now, because we are able to consistently produce preretinal neovascularization, we can begin to test the effectiveness of blood vessel growth inhibitors for the first time in the context of ROP. If a pharmacologic means (eg, antioxidant supplementation, anti-angiogenic agents) of arresting either the initial attenuation or the subsequent proliferation of retinal vessels can be demonstrated in animal models, the same therapeutic strategy can be made available to infants at risk for ROP.

Key Words

ADPase staining, preretinal neovascularization, rat, retinopathy of prematurity, variable oxygen
Preretinal Vessels in the Hyperoxic Rat

We wish to thank J. Bunch for processing the manuscript and A. Bandyopadhyay for technical assistance. We are grateful to K. D’Arezzo, who critically reviewed the manuscript.

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